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The effect of cysteine on the altered expression of class α and μ glutathione *S*-transferase genes in the rat liver during protein–calorie malnutrition

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Abstract

Protein–calorie malnutrition (PCM) represents a global health problem. The breakdown rate of glutathione *S*-transferase (GST) subunits determines their differential contents during protein depletion. Hepatic GST expression and the underlying mechanistic basis were investigated in PCM rats. PCM caused no change in rGSTA1/2 subunit. In contrast, rGSTA3/5 subunit was 2.4-fold induced during PCM, while the levels for rGSTM1 and M2 subunits were 30% and 70% suppressed. Increased GSTA3/5 expression was significantly prevented by cysteine or methionine treatment, although such treatment failed to restore the rGSTM2 level. In contrast to differential GST protein expression, PCM caused a 5–10-fold increase in rGSTA2/A3/A5 and M1 mRNAs, whereas rGSTM2 mRNA was 70% decreased. The elevations in rGSTA2/A3/A5 and M1 mRNAs were completely abolished by cysteine or methionine treatment during PCM, although the rGSTM2 mRNA level was not restored. PCM induced oxidative stress in the liver, as evidenced by protein carbonylation. Antioxidant response element (ARE)-binding activity of nuclear extracts from PCM rats was increased, which was immunodepleted with anti-Nrf-1/2 antibodies. Activation of nuclear ARE-binding proteins was inhibited by cysteine. Data showed that hepatic GSTs were differentially expressed during PCM, that certain GST mRNAs were increased with the ARE activation, and that cysteine was active in preventing increases in GST mRNAs and ARE activation.

Keywords: Protein–calorie malnutrition; Nrf-1/2; Cysteine; Methionine; Glutathione *S*-transferase; Antioxidant response element

1. Introduction

Protein–calorie malnutrition (PCM) represents a significant global health problem affecting developing and developed countries. PCM arises when there is insufficient energy or protein available to meet metabolic demands due to disease and increased nutrient losses [1]. The rapidity of PCM development is determined by the factors such as nutritional adequacy, underlying diseases and physiologic states [1]. A low content of certain essential amino acid(s) may become a limiting factor for PCM.

Changes in the basal metabolism and energy utilization have been studied as part of compensatory responses functioning during protein and/or energy deficit [2]. The physiology of alterations of drug me-

Abbreviations: EDTA, ethylenediamine tetraacetic acid; PCM, protein–calorie malnutrition; GST, glutathione *S*-transferase; ARE, antioxidant response element; SDS, sodium dodecyl sulfate; SSC, standard saline citrate

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tabolism should be investigated for PCM patients taking medication therapy. Studies from our laboratories have shown that PCM influences pharmacokinetics and pharmacodynamics of therapeutic agents [3,4]. PCM suppresses the expression of major cytochrome P450s in the liver [5].

Glutathione *S*-transferase (GST) enzymes are involved in the metabolism of a wide variety of electrophilic substrates including therapeutic agents and toxicants. It has been shown that the activities of the cellular proteolytic system are increased during protein deficiency [6,7] and that translational efficiency would be decreased. Hence, the breakdown rate of GST subunits determines the differential contents during protein depletion [8]. In addition, the gene regulation and protein synthesis rate would contribute to the altered expression of the detoxifying enzymes. Protein restriction decreases the hepatic glutathione content [9], which would enhance oxidative stress and stimulate the associated gene expression. Previous studies have shown that class α GSTs are transcriptionally activated in response to oxidative stress [10,11]. Thus, the regulatory mechanism for the expression of GST appears to be complicated during PCM. In view of the complex effects of protein restriction on the turnover of GST and the effect of oxidative stress on GST gene expression, we were interested in determining whether a 4 week protein restriction changes the expression of GST subunits and of the major GST genes in the liver. Efforts were made to find the amino acid(s) primarily responsible for the regulation of GST gene expression during PCM. Because cysteine prevents toxicant-induced liver injury apparently through the mechanism involving elevation of intracellular GSH levels [12], the effect of sulfur-containing amino acid, a potential limiting factor, on GST gene expression was investigated during PCM.

2. Materials and methods

2.1. Materials

[α-^{32}P]dCTP (3000 mCi/mmol) and [γ-^{32}P]ATP (3000 mCi/mmol) were purchased from New England Nuclear (Arlington Heights, IL, USA). The formulated isocaloric diets containing 5% or 23% casein were supplied from Dong-A Pharmaceutical Co. (Yongin, South Korea), as described previously [5]. Anti-rGSTA1/2, anti-rGST3/5, anti-rGSTM1 and anti-rGSTM2 antibodies were supplied from Biotrin International (Dublin, Ireland). Biotinylated goat anti-rabbit IgG, recombinant protein G-agarose and 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium were obtained from Life Technologies (Gaithersburg, MD, USA). Anti-Nrf-1, anti-Nrf-2 and anti-v-Maf antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Random prime-labeling kit was purchased from Promega (Madison, WI, USA). Anti-dinitrophenyl antibody and most of the reagents in the molecular studies were supplied from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Methods

2.2.1. Animal treatment

Male Sprague–Dawley rats (150–190 g) were purchased from Charles River Co. (Atsugi, Japan) and maintained in a clean room at the Animal Center for Pharmaceutical Research, College of Pharmacy, Seoul National University, at a temperature between 20°C and 23°C with 12 h light and dark cycles and a relative humidity of 50%. Animals were caged under the supply of filtered pathogen-free air and water ad libitum. Rats at 5 weeks of age were randomly assigned to two groups that were fed either the control diet containing 23% casein or the low protein diet containing 5% casein for 4 weeks. Food intake and body weights were recorded at least once a week [5]. Animals were gavaged with either cysteine or methionine at the daily dose of 500 mg/kg body weight (250 mg/kg body weight twice per day) for the last 7 days during 4 weeks of protein restriction. Cysteine was dissolved in an aqueous solution, whereas methionine was suspended in 0.1% carboxymethylcellulose.

2.2.2. Cysteine and cystine contents

The contents of plasma cysteine and cystine were measured according to the previously published methods [13,14]. Two hundred μl of plasma was mixed with 100 μl of ice-cold 10% 5-sulfosalicylic acid and centrifuged at 3000×*g* for 10 min at 4°C to obtain supernatant. Cysteine was assayed with 150 μl of the supernatant, which was mixed with the

same volumes of 100% glacial acetic acid and of the acid ninhydrin reagent, and the mixture was heated at 110°C for 10 min. The acid ninhydrin reagent was freshly prepared by mixing 6 ml of 100% glacial acetic acid, 4 ml of 35% hydrochloric acid and 250 mg of ninhydrin. After rapid cooling, 1 ml of 95% ethanol was added to the mixture and left at room temperature for 30 min. The absorption was spectrophotometrically determined at 560 nm. The plasma cystine level was also assessed in a similar way with 100 μl of the supernatant, which was mixed with 110 μl of 0.5 M Tris–Cl buffer (pH 8.0) containing 10 μl of 0.3% phenolphthalein dissolved in 70% ethanol. The mixture was left at room temperature for 30 min after addition of 20 μl of 0.1 M dithiothreitol, and the content of cystine was spectrophotometrically assayed.

2.2.3. Hepatic GSH content

The GSH content was measured using a commercial kit according to the manufacturer's protocol (GSH-400 method, Oxis International, Portland, OR, USA) using 4-chloro-1-methyl-7-trifluoromethyl-quinolinum methylsulfate.

2.2.4. GST assay

The activity of cytosolic GST was measured with 1-chloro-2,4-dinitrobenzene as a substrate as described by Habig et al. [15]. Incubation mixtures contained 25 μg of cytosolic proteins, 1 mM 1-chloro-2,4-dinitrobenzene, 1 mM reduced GSH and 0.1 M potassium phosphate buffer, pH 6.5, in a total volume of 1 ml.

2.2.5. Immunoblot analysis

Hepatic cytosolic fractions were prepared by differential centrifugation. The subcellular preparations were stored at −70°C until used. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and immunoblot analysis were performed according to previously published procedures [16,17]. Cytosolic proteins were separated by 12% gel electrophoresis and electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with form-specific anti-rat GST antibodies, followed by incubation with biotinylated secondary antibody, and developed using 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium [17]. Specificity of the antibodies for GST subunits has been confirmed by a series of previous studies [16–20].

2.2.6. Preparation of cDNA probes for GST

Specific cDNA probes for major GST genes were amplified by reverse transcriptase-polymerase chain reaction using the selective primers [16–19] and were cloned in the pGEM+T vector (Promega, Madison, WI, USA).

2.2.7. Northern blot hybridization

Total RNA was isolated using the improved single-step method of thiocyanate–phenol–chloroform RNA extraction, as described by Puissant and Houdebine [21]. Northern blot analysis was carried out according to the procedures described previously [16]. Briefly, total RNA isolated from rat livers was resolved by electrophoresis in a 1% agarose gel containing 2.2 M of formaldehyde and transferred to nitrocellulose paper. The nitrocellulose paper was baked in a vacuum oven at 80°C for 2 h. The blot was incubated with hybridization buffer containing 50% deionized formamide, 5×Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone and 0.1% bovine serum albumin (Pentex Fraction V)), 0.1% SDS, 200 μg/ml of sonicated salmon sperm DNA and 5×SSPE (1×SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4 and 1 mM Na_2EDTA (ethylenediamine tetraacetic acid), pH 7.4) at 42°C for 1 h without probe. Hybridization was performed at 42°C for 18 h with a heat-denatured cDNA probe, which was random prime-labeled with [α-^{32}P]dCTP. Filters were washed in 2×standard saline citrate (SSC) and 0.1% SDS for 10 min at room temperature twice and in 0.1×SSC and 0.1% SDS for 10 min at room temperature twice. Filters were finally washed in the solution containing 0.1×SSC and 0.1% SDS for 60 min at 60°C. After quantitation of mRNA levels, the membranes were stripped and rehybridized with a ^{32}P-labeled cDNA probe complementary to 18S rRNA to quantify the amount of RNA loaded onto the membranes.

2.2.8. Data analysis

Scanning densitometry was performed with a Microcomputer Imaging Device, Model M1 (Imaging Research, St. Catharines, Ont., Canada). One way analysis of variance procedures were used to assess

significant differences among treatment groups. For each significant effect of treatment, the Newman–Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $P < 0.05$ or $P < 0.01$.

2.2.9. Gel retardation assay

A double-stranded DNA probe containing the rGSTA2 gene antioxidant response element (ARE) was used for gel shift analysis after end-labeling of the probe with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The sequence of the ARE-containing oligonucleotide was (5′-GATCATGGCATTGCACTAGGTGACAAAGCA-3′). The oligonucleotides for SP-1 and AP-1, which were used as negative controls for a competition experiment, were (5′-ATTCGATCGGGGCGGGGCGAGC-3′) and (5′-CGCTTGATGAGTCAGCCGGAA-3′), respectively. Nuclear extracts were obtained by a modification of the procedure published previously [22]. The reaction mixtures contained 2 μl of 5× binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dI-dC and 50 mM Tris–Cl (pH 7.5), 3.5 μg of nuclear extracts and sterile water in a total volume of 10 μl. The reaction mixtures were preincubated for 10 min. DNA-binding reactions were carried out at room temperature for 20 min after addition of 1 μl probe (10^6 cpm). Specificity of binding was determined by a competition experiment, which was carried out by adding a 5- or 20-fold excess of an unlabeled ARE, SP-1 or AP-1 oligonucleotide to the reaction mixture before the DNA-binding reaction. Samples were loaded onto 4% polyacrylamide gels at 100 V. The gels were removed, fixed and dried, followed by autoradiography.

In some experiments, 2 μg of nuclear extract proteins was incubated with 2 μg of highly specific anti-Nrf-1, anti-Nrf-2 or anti-v-Maf antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on ice for 1 h. For immunodepletion, 15 μl of a 1:1 slurry of recombinant protein G-agarose (Life Technologies Inc., Gaithersburg, MD, USA) was incubated with the nuclear extracts for 60 min. The immune complexes were removed by centrifugation, and the nuclear extract was assayed for ARE-binding activity by electrophoretic mobility shift assay [23].

2.2.10. Determination of protein carbonyl groups

Protein carbonylation was determined according to the previously described method [24,25]. Liver microsomal fractions were electrophoretically separated by 7.5% polyacrylamide gels and transferred to nitrocellulose paper. The nitrocellulose paper was incubated with 0.5 mM of 2,4-dinitrophenylhydrazine, which reacts with carbonyl groups formed as a consequence of protein oxidation, after incubation with 5% non-fat dried milk at 4°C overnight. The membrane was washed three times and incubated for 1 h with an anti-dinitrophenyl antibody (1:2500, Sigma Chemical Co.). The membrane was incubated for 1 h with an alkaline phosphatase-conjugated anti-rabbit antibody (1:5000), followed by incubation with biotinylated secondary antibody. The color was developed using 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium [17].

3. Results

3.1. Expression of hepatic GST during PCM

The conjugating activity of GST toward 1-chloro-2,4-dinitrobenzene produced from PCM rats was 28% decreased in the hepatic cytosol, as compared to control (1.04 ± 0.25 vs. 0.75 ± 0.15 μmol/min/mg proteins, significant at $P < 0.01$, $n = 4$). This was in agreement with the previous study [26].

The major GST subunits were immunochemically monitored in the liver cytosol (Fig. 1). The rGSTA1/2 protein expression was not changed by 4 weeks of protein deprivation. In contrast, rGSTA3/5 subunit was 2.4-fold induced, relative to control (Table 1). The levels for rGSTM1 and rGSTM2 subunits were 30% and 70% suppressed in PCM rats, respectively. Hence, the major GST subunits were differentially expressed during PCM, despite the decrease in GSH-conjugating catalytic activity toward 1-chloro-2,4-dinitrobenzene.

To determine whether changes in the levels of the major GST subunits paralleled those of GST mRNAs, Northern blot analysis was performed using the specific GST cDNA probes. Fig. 2 shows the major GST mRNA levels monitored in the rat liver during PCM. In contrast to the differential changes

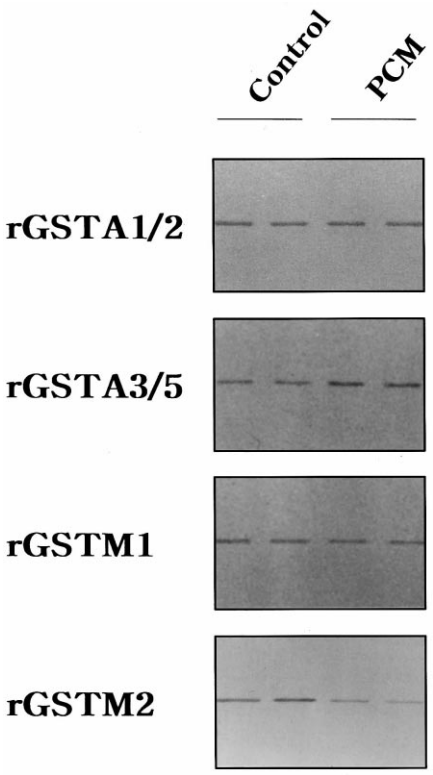

Fig. 1. Immunoblot analyses of hepatic GST proteins. The immunoblots show GST protein levels in the liver cytosol isolated from rats fed the 23% (control) or 5% (PCM) casein diet for 4 weeks. Each lane was loaded with 5 μg of proteins.

in GST protein levels, rGSTA2, A3, A5 and M1 mRNAs were 10-, 6-, 10- and 5-fold increased in the livers of PCM rats, respectively, as compared to the respective control (Table 2). Conversely, the rGSTM2 mRNA was 70% decreased (Fig. 2 and Table 2).

3.2. Activation of nuclear ARE-binding protein(s)

Previous studies have shown that ARE-binding activity regulates expression of the rGSTA2 and A5 genes in response to hydrogen peroxide and phenolic antioxidants [11,27–29]. Activation of ARE also upregulates the expression of γ-glutamylcysteine synthetase [30]. Studies were extended to determine whether the increased GST mRNA levels during PCM accompany activation of ARE. Electrophoretic mobility shift assay revealed that the ARE DNA-binding activity was increased in nuclei from PCM rats. Three distinct bands were detected when the ARE probe was incubated with the nuclear extracts isolated from PCM rat livers. Two slower migrating band complexes were clearly increased during PCM (Fig. 3A). Specificity of the DNA probe to the PCM-activated ARE-binding complex was supported by competition for binding to a radiolabeled ARE probe with a 5–20-fold molar excess of unlabeled ARE oligonucleotide, but not with an excess of unlabeled SP-1 or AP-1 oligonucleotide (Fig. 3A). To confirm the factors that make up the inducible ARE activity immunochemically, the highly specific antibodies directed against Nrf-1, Nrf-2 and v-Maf were evaluated for the ability to inhibit the DNA-binding activity (Fig. 3B). Immunodepletion experiments showed that anti-Nrf-1 antibody was effective at depleting the slowest migrating ARE-binding complex,

Table 1
Major hepatic GST protein levels in PCM rats with or without sulfur amino acid supplementation

Subunits	Treatment					
	Untreated		Cysteine		Methionine	
	−PCM	+PCM	−PCM	+PCM	−PCM	+PCM
rGSTA1/2	1	1.2 ± 0.4	1.1 ± 0.2	0.9 ± 0.3	1.2 ± 0.2	0.7 ± 0.2
rGSTA3/5	1	2.4 ± 0.3**	0.8 ± 0.4	1.5 ± 0.4*,‡	0.8 ± 0.4	1.3 ± 0.5*,‡
rGSTM1	1	0.7 ± 0.1**	0.9 ± 0.1	0.6 ± 0.2**	1.0 ± 0.1	0.6 ± 0.1**
rGSTM2	1	0.1 ± 0.03**	0.9 ± 0.4	0.1 ± 0.05**	1.1 ± 0.4	0.1 ± 0.04**

The relative changes in hepatic GST protein levels were quantified in rats fed the 23% (−PCM) or 5% (+PCM) casein diet for 4 weeks. Cysteine or methionine (250 mg/kg body weight twice per day) was supplemented for the last 7 days during the 4 week period. Band intensities were determined by scanning densitometry of the immunoblots. Data represent the mean ± S.D. with six separate experiments. One way analysis of variance was used for comparisons of multiple group means followed by Newman–Keuls test (significant as compared to rats fed the normal diet, $*P < 0.05$, $**P < 0.01$; significant as compared to rats fed the protein-deficient diet, $^{‡}P < 0.01$) (control level in rats fed the normal diet = 1).

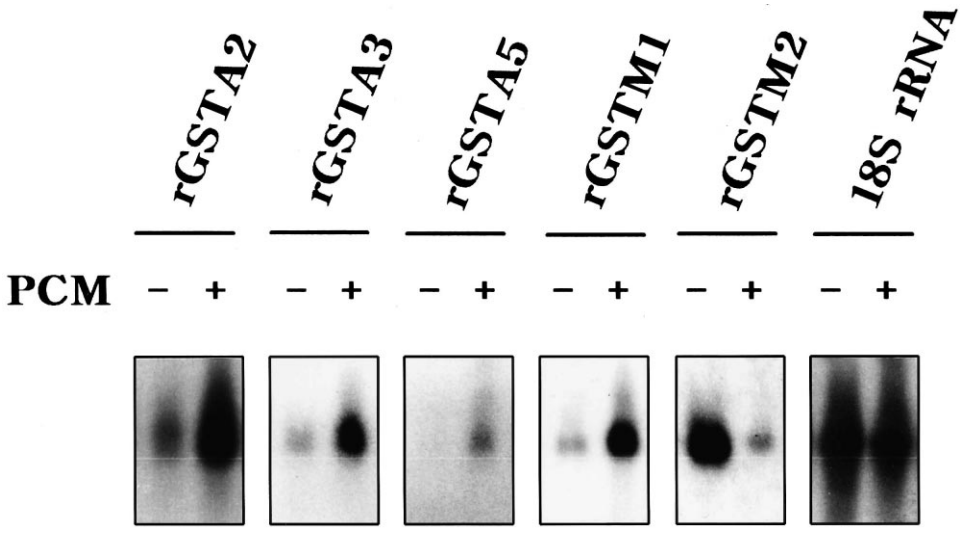

Fig. 2. Northern blot analyses for GST mRNAs in PCM rats. The GST mRNA levels were determined in total RNA fractions (20 μg each) isolated from rats fed the 23% (−PCM) or 5% (+PCM) casein diet for 4 weeks. The amount of RNA loaded in each lane was assessed by rehybridization of the stripped membrane with a ^{32}P-labeled probe for 18S rRNA. Relative changes in the GST mRNA levels were assessed by scanning densitometry of Northern blots.

whereas anti-Nrf-2 and anti-v-Maf antibodies were less active (Fig. 3B).

3.3. Protein carbonylation by PCM

Protein carbonyl groups, formed as a result of oxidative damage, were detected in the hepatic microsomal fractions prepared from PCM rats to determine whether PCM elicited oxidative stress [24,25]. Western immunoblot analysis using anti-dinitrophenyl antibody revealed that the extents of band intensities were much greater in the livers of PCM rats than those in control animals (Fig. 4). The result of carbonyl assay supported the notion that PCM increased oxidative stress in the liver.

3.4. Plasma cysteine/cystine and hepatic GSH contents after protein deficiency

The plasma contents of cysteine and cystine were assessed in PCM rats. Cysteine and cystine contents were 30% and 65% decreased during PCM, as com-

Table 2
Hepatic GST mRNA levels in PCM rats with or without cysteine/methionine supplementation

Subunits	Treatment					
	Untreated		Cysteine		Methionine	
	−PCM	+PCM	−PCM	+PCM	−PCM	+PCM
rGSTA2	1	9.9 ± 2.4**	1.4 ± 1.2	1.0 ± 0.7‡	1.2 ± 0.8	2.6 ± 0.7‡
rGSTA3	1	6.0 ± 1.2**	0.8 ± 0.3	1.9 ± 1.1‡	0.9 ± 0.3	3.3 ± 1.0‡
rGSTA5	1	10.3 ± 2.3**	0.8 ± 0.3	2.0 ± 1.1‡	1.4 ± 0.9	3.2 ± 1.4‡
rGSTM1	1	5.1 ± 1.2**	0.9 ± 0.2	0.6 ± 0.3‡	0.7 ± 0.3	0.7 ± 0.2‡
rGSTM2	1	0.3 ± 0.4**	0.8 ± 0.3	0.4 ± 0.5**	1.3 ± 0.1	0.5 ± 0.5**

The hepatic GST mRNA levels were assessed in rats fed the 23% (−PCM) or 5% (+PCM) casein diet for 4 weeks. Changes in the hepatic GST mRNA levels were assessed by scanning densitometry of the Northern blots. Data represent the mean ± S.D. with six separate experiments. One way analysis of variance was used for comparisons of multiple group means followed by Newman–Keuls test (significant as compared to rats fed the normal diet, $^{**}P < 0.01$; significant as compared to rats fed the protein-deficient diet, $^{‡}P < 0.01$) (control mRNA level = 1).

pared to control, respectively (Table 3). The hepatic GSH content was also decreased to 1.62 μmol/g liver wet weight after 4 weeks of protein restriction from 4.17 μmol/g liver wet weight in control rats. The reduction in the GSH content by PCM was in agreement with marked decreases in the plasma cysteine and cystine levels.

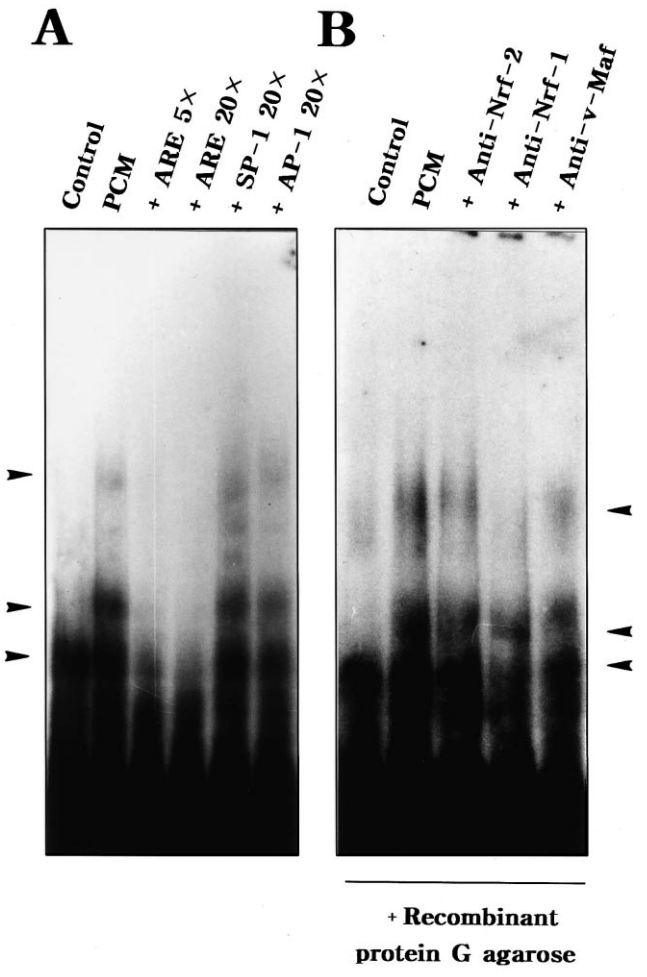

Fig. 3. Gel shift analysis of the ARE transcription factor complexes in hepatic nuclear extracts. (A) Hepatic nuclear extracts were isolated from rats fed a 23% casein diet (control) or rats fed a 5% casein diet (PCM). All lanes contained 3.5 μg of nuclear extracts and 5 ng of radiolabeled ARE consensus sequence. Competition studies were carried out by adding a 5- or 20-fold excess of an unlabeled ARE, SP-1 or AP-1 oligonucleotide before DNA-binding reactions. (B) Immunodepletion experiments were carried out by incubating the nuclear extracts from PCM rats with the polyclonal antibodies directed against Nrf-1, Nrf-2 and v-Maf proteins. Results were confirmed by repeated experiments.

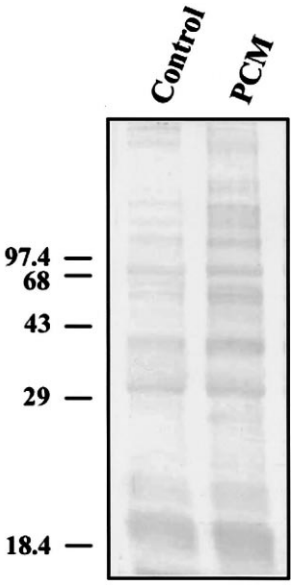

Fig. 4. Protein carbonylation in PCM rats. Western blot analysis was carried out with hepatic nuclear extracts prepared from control or PCM rats. Oxidatively damaged hepatic proteins reacted with dinitrophenylhydrazine were assessed by an anti-dinitrophenyl antibody. Molecular weights (kDa) were indicated. Results were confirmed by multiple analyses.

3.5. *Effects of cysteine supplementation on GST expression*

An additional study was carried out to determine whether cysteine supplementation affected GST expression. Cysteine supplementation caused no significant changes in the levels of GST subunits in rats fed 23% casein diet. Interestingly, the induction of rGSTA3/5 subunit was significantly prevented by cysteine in PCM rats (Table 1). The rGSTA1/2 protein level was rather slightly decreased by the sulfur amino acid. Methionine was as effective as cysteine at the same dose. The decreased rGSTM1 and M2 levels during PCM failed to be restored by the sulfur amino acids (Table 1).

Northern blot analysis was performed whether the sulfur amino acids altered rGST mRNAs in PCM rats. The elevations of rGSTA2/A3/A5 and rGSTM1 mRNAs were completely abolished by cysteine supplementation during PCM (Fig. 5 and Table 2). However, the suppressed rGSTM2 mRNA level was not restored (Fig. 5 and Table 2). The effects of methionine were comparable to those of cysteine. These results showed that sulfur amino acids were

Table 3
The plasma cysteine and cystine contents in PCM rats with or without cysteine supplementation

Plasma concentration (μM)	Treatment			
	Control	Control+cysteine	PCM	PCM+cysteine
Cysteine	87.4 ± 10.8	80.5 ± 2.6	60.4 ± 0.9*	91.3 ± 7.6†
Cystine	66.4 ± 11.8	70.4 ± 8.2	23.1 ± 1.7**	46.9 ± 6.3‡

Data represent the mean ± S.D. with three separate experiments. One way analysis of variance was used for comparisons of multiple group means followed by Newman–Keuls test (significant as compared to control, $*P < 0.05$, $**P < 0.01$; significant as compared to PCM, $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$).

active in preventing the elevations in GST mRNA levels, but not in restoring the suppressed GST expression.

3.6. Cysteine effect on ARE activation

Gel shift analysis revealed that the specific increase in the nuclear ARE complex was completely abolished by cysteine supplementation during PCM, which indicates that ARE activation during PCM resulted from the deficiency of cysteine (Fig. 6).

3.7. Effect of cysteine on PCM-induced protein carbonylation

The effect of cysteine on the extent of protein carbonylation was assessed in PCM rats. The band intensities increased during PCM failed to be decreased by a week of cysteine supplementation, as shown by Western immunoblots (Fig. 7). Data indicate that protein carbonylation caused by PCM was not reversible by the late cysteine supplementation.

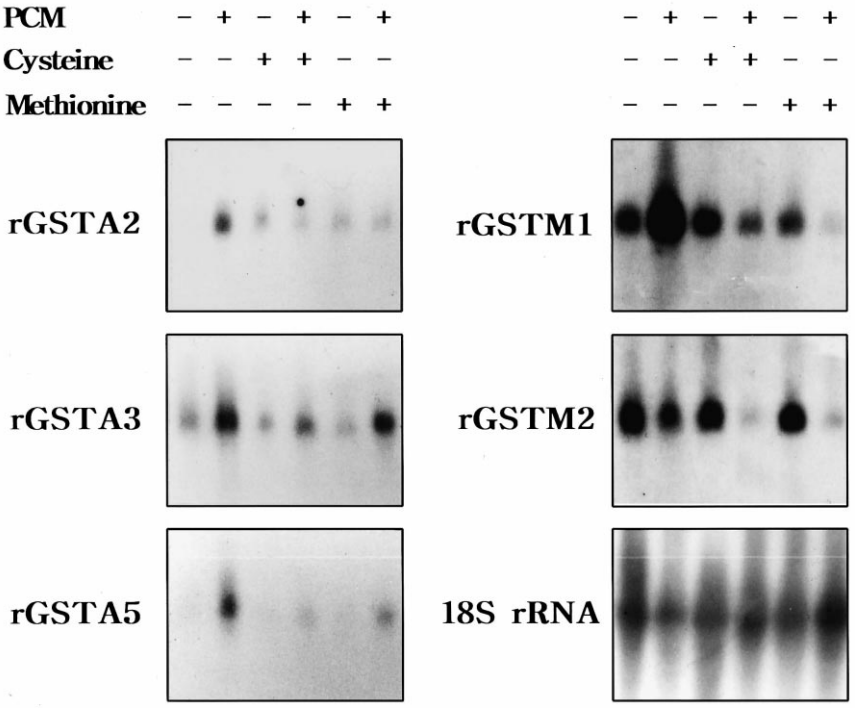

Fig. 5. The hepatic GST mRNA levels in PCM rats with or without sulfur amino acid supplementation. Northern blot analyses for the GST mRNAs were carried out with total RNA fractions (20 μg each) isolated from rats fed the 23% (−PCM) or 5% (+PCM) casein diet for 4 weeks with or without cysteine/methionine supplementation. Cysteine or methionine was administered for the last 7 days during 4 weeks of protein restriction.

3.8. The plasma cysteine/cystine and hepatic GSH contents after cysteine supplementation

Cysteine supplementation at the dose of 250 mg/kg twice a day for 1 week completely prevented PCM-induced reduction in the plasma cysteine content (105% of control) (Table 3). The cystine level was also significantly restored by cysteine supplementation to 70% of control. We also measured the hepatic GSH content. A week of cysteine supplementation to PCM rats returned the GSH level to 7.15 μmol/g liver wet weight, which was even greater than control (5.93 μmol/g liver wet weight).

4. Discussion

The degradation rate, half-life and synthesis rate of cytosolic GST subunits are greatly affected in mice during protein restriction [8]. The results in the present study showed the differential expression of major GSTs in the rat liver during PCM. The subunit for rGSTA2 was not significantly changed. The rGSTA3/5 was induced, whereas rGSTM1 and M2 were suppressed. The induction of rGSTA3/5 was consistent with the previous study showing that the half-life of Yc subunit (the homologous form of rGSTA3/5) was greater than those of other forms during protein depletion [8]. In contrast to the differential expression of GST proteins, rGSTA2/A3/A5 and rGSTM1 mRNAs were markedly increased during PCM. A previous study has shown that the synthesis rate of mouse GSTM1 and GSTA3/5 was 50% and 26% increased, respectively, in the state of protein deficiency [8]. This is in agreement with the increases in rGSTM1 and rGSTA3/5 mRNAs in the present study. The slight suppression of rGSTM1 protein level may represent the rapid breakdown of the GST subunit. Thus, the discrepancy between protein and mRNA expression for rGSTA1/2 and rGSTM1 would result from the different breakdown rate for each GST subunit. The expression of

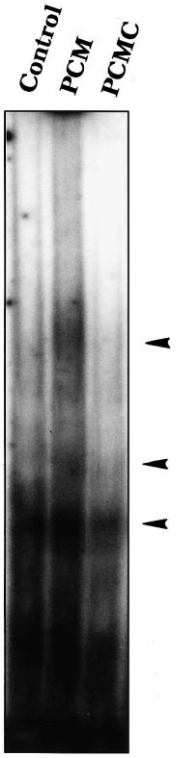

Fig. 6. Effect of cysteine on the ARE transcription complexes during PCM. Gel shift analysis was carried out with hepatic nuclear extracts from PCM rats supplemented with cysteine in vivo (250 mg/kg body weight twice per day) for 7 days. These results were confirmed by multiple experiments.

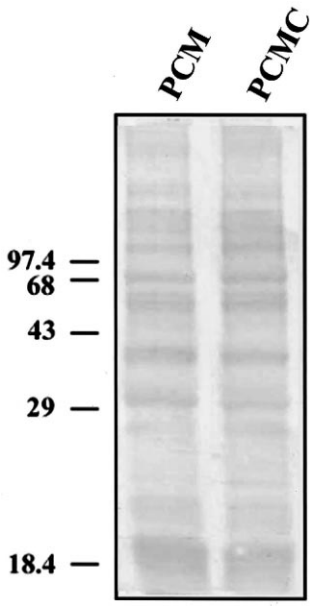

Fig. 7. Protein carbonylation in PCM rats with or without cysteine supplementation. Hepatic microsomes produced from PCM rats with or without cysteine supplementation (250 mg/kg body weight twice per day, for 7 days) were subjected to the Western blot analysis. Oxidatively damaged hepatic microsomal proteins were reacted with dinitrophenylhydrazine and immunoblotted with an anti-dinitrophenyl antibody. Molecular weights (kDa) were indicated.

rGSTM2 was distinctly suppressed (90%) during PCM, as compared to control, which was in parallel with the suppression in the rGSTM2 mRNA. Hence, the regulatory mechanism of rGSTM2 gene expression appeared to differ from that of other GSTs.

Studies have shown that protein depletion caused an extensive breakdown of hepatic polysomes [31]. Hence, PCM reduced the translational efficiency of the plasma proteins (e.g. albumin, retinol-binding protein and prothrombin) and insulin-like growth factor, as evidenced by a change in the polyribosomal distribution profile [32,33]. The altered rate of protein synthesis may be related in part with the increase in the mRNAs. It is likely that the expression of major GSTs during PCM is affected by the rates of protein translation and proteolytic degradation.

The present study showed that the plasma cysteine and cystine contents were markedly decreased during PCM, whereas cysteine supplementation reversed PCM-induced reduction in the plasma cysteine and cystine. Although cysteine conjugates with toxicants might exert toxic effects in animals [34], orally administered cysteine per se caused no toxicity. This was consistent with the previous no report on toxicity by cysteine. Depletion of hepatic GSH increases the susceptibility of animals to free radical-induced tissue damage because liver GSH plays a critical role in the detoxification of oxidative metabolites produced from endogenous and exogenous molecules. The hepatic GSH content decreases after low protein diet [9]. The present study also showed that the plasma cysteine and cystine contents were markedly decreased with a concomitant reduction in the hepatic GSH by 4 weeks of protein deprivation. Because cysteine is a direct precursor of GSH, hepatic GSH content was increased by cysteine supplementation in rats fed either the normal protein diet or protein-deficient diet. The greater increase in hepatic GSH content was observed in PCM rats. We further determined the effect of sulfur amino acids on the GST expression. The hepatic GST expression was not significantly altered by cysteine in rats fed the 23% casein diet. The PCM-induced decrease ($\sim$27%) in cytosolic GST catalytic activity toward 1-chloro-2,4-dinitrobenzene was not restored by cysteine supplementation. Cysteine and methionine, however, were effective in preventing elevations in the GST mRNA levels during PCM, but not in restoring the suppressed GST mRNA. The distinct regulation of rGSTM2 expression by PCM was in parallel with no recovery of rGSTM2 mRNA level after cysteine supplementation. Reversal of GST mRNA expression by cysteine was also observed at the daily dose of 50 mg/kg. Changes in the enzyme expression seem to be affected by the GSH content in the liver. However, it is also possible that cysteine directly affects the sulfhydryl residues of critical proteins associated with the gene regulation [35,36].

Oxidative stress induces carbonylation of amino acid residues in proteins (e.g. histidine, arginine, lysine and proline). The present study clearly demonstrated that protein carbonylation was increased in the hepatic microsomes prepared from PCM rats, which indicated that the microsomal proteins were oxidatively damaged as a consequence of reactive oxygen species. The cellular redox state affects transactivation of oxygen-responsive genes [27,37,38]. The enzyme induction by protein restriction as well as prevention of the induction by sulfur amino acids also lends support to the notion that PCM elicits oxidative stress in hepatocytes, which alters GST gene expression. Transcriptional activation of the GST genes may be associated with the change in the redox state in conjunction with oxidative stress and with reduction in the intracellular GSH level. Transcriptional activation of hepatic rGSTA2 and rGSTA5 genes in response to phenolic antioxidants is mediated by ARE [11,28]. Thus, studies were extended to determine the molecular basis for the increases in GST mRNAs during PCM. The present study clearly demonstrated that the nuclear ARE complexes were activated in the livers of PCM rats. Previous studies have shown that nuclear transcription factors Nrf-1 and Nrf-2 associate with Jun (c-Jun, JunB and JunD) and small Maf proteins to upregulate ARE-mediated expression and coordinated induction of detoxifying enzymes [39,40]. Small Maf proteins as bZIP transcription factors strongly bind to the consensus Maf recognition sequence and form heterodimers with Nrf, Jun and Fos proteins. It has been shown that Maf proteins increase the specificity of DNA-binding and transactivation activity [41]. The observation that the ARE-binding activity was reduced by incubation of the nuclear extracts with the antibodies directed against Nrf-1/2 and

Maf proteins provided evidence that the nuclear proteins are involved in the ARE activation during PCM. The results of the present study support the conclusion that oxidative stress induced by PCM activates Nrf proteins, which would be responsible for the transcriptional activation of the GST genes [39]. The conclusion that PCM elicits oxidative stress was further supported by the reversal of ARE activation in response to cysteine supplementation. Although the genomic DNA for rGSTM2 has not been characterized in rats yet, the homologous mouse GST gene was shown to have no exact match to the ARE [42]. This would explain the distinct rGSTM2 expression in PCM rats.

Results of this study demonstrated that PCM elevates rGSTA2, rGSTA3/5 and rGSTM1 mRNAs in association with activation of ARE-binding proteins, which may result from oxidative stress in hepatocytes, and that expression of the GST subunits during PCM is not always consistent with the change in the mRNAs. This would be due to the differential breakdown rate for each GST subunit and the altered translational efficiency.

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